ORIGINAL RESEARCH

A Sabin 1 poliovirus-based vaccine vector transfects Vero cells with high efficiency

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Abstract Over the past 40 years, live oral poliovirus (PV) vaccines have contributed to the eradication of wild PV in most countries. These live vaccine strains have a high safety record and can stimulate both cellular and humoral immune responses. As both of these factors are critical characteristics of a good vaccine, we aimed to modify the oral PV vaccines to create a powerful vaccine vector for extraneous antigen expression. In this study, we amplified three separate fragments from the Sabin 1 virus genome by RT-PCR and cloned them into the pGEM-TEasy vector. A cassette containing engineered protease cleavage sites and a polylinker was introduced into one of these fragments (f1) in front of the translation start site. This construction facilitated the insertion of foreign genes into the vector and the subsequent release of their co-translated antigens after digestion by endogenous protease. We also placed a ribozyme (Rz) sequence between the T7 promoter and viral genomic DNA so that in vitro transcription and Rz cleavage recreated the authentic $5'$ end of the PV genome RNA. Poly(A)$_{40}$ tails were added to the $3'$ end of the genome to stabilize the transcribed RNA. The three PV genome fragments and their derivatives were cloned into various types of vectors that were transfected into Vero cells. Virus rescue experiments demonstrated that both the Rz and poly(A)$_{40}$ elements were required for high transfection efficiency of the vector-derived RNAs.

Keywords Ribozyme · Sabin 1 virus · Transfection · Vaccine vector · Vero cell

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Introduction

Live-attenuated vaccines are powerful weapons for combating human viral diseases, and a number of them are in routine use. Live vaccines provoke both local and systematic immune responses. Both innate and adaptive immunity are involved in stimulating humoral and cell-mediated responses (Levine et al. 2004). The widespread administration of the Sabin 1, 2, and 3 vaccine strains of poliovirus (PV) has brought PV to the brink of global eradication. The Sabin strains are safe and easy to manipulate experimentally, and their low cost makes their production and distribution affordable, which is a particularly

important concern in developing countries. Because of these factors, the Sabin PV strains are ideal vaccine vectors for foreign antigen expression.

Various strategies have been employed to modify Sabin viruses into versatile vaccine vectors. Small epitopes can be conjugated to one of the neutralizing domains of the capsid proteins (Burke et al. 1989; Dedieu et al. 1992), but the usefulness of this hybrid virion strategy is greatly decreased by restrictions on the size of the attached epitope (normally < 25 amino acids). Another strategy, in which one of the structural protein genes is replaced with the foreign gene (Bledsoe et al. 2000; Novak et al. 1999), results in recombinant viruses that are defective in replication and require a helper virus to provide the necessary structural protein. In the most frequently adopted strategy, polyprotein fusion, the foreign antigen is fused at either the N-terminus of the polyprotein or at the junction between the capsid proteins and the nonstructural proteins in the PV polyprotein, along with an artificial protease site. Thus, after co-translation with the viral polyprotein, the foreign antigen is easily removed by one of the endogenous proteases and is released into the cytoplasm (Andino et al. 1994; Mattion et al. 1995).

In previous attempts to use the polyprotein fusion strategy, the PV genome was inserted into a plasmid carrying the T7 promoter. RNA was transcribed on a large scale from a linearized plasmid template in the presence of T7 polymerase. The recombinant virus was generated by introducing the infectious RNA into a permissive cell line. However, the transcripts prepared by this method possessed two non-native 5′-terminal G nucleotides that had no effect on minus-strand synthesis but caused plus-strand RNA synthesis to decrease to an undetectable level (Barton et al. 2002; Morasco et al. 2003). Also, the precise 5′ end has been shown to be essential for efficient plus-strand RNA synthesis (Herold and Andino 2000).

In the present study, we placed a hammerhead ribozyme (Rz) sequence between the T7 promoter and the 5′ end of the PV sequence in the vectors, as described by Herold and Andino (2000). After transcription in vitro, the Rz was removed by *cis*-active cleavage, releasing the genome RNA with the correct 5′ end. Virus rescue experiments proved that this Rz motif, along with the poly(A)$_{40}$ stretch, is essential for high transfection efficiency of vector-derived RNAs in Vero cells.

In this work, we investigated the utility of our PV vector constructs for expression of foreign antigens. Two foreign genes, CTBM2e (encoding a hybrid of the B subunit of cholera toxin from *Vibro cholerae* and the M2 extracellular domain from type A influenza virus) and SARS-RBD (encoding the receptor-binding domain (RBD) of the spike (S) protein of the severe acute respiratory syndrome (SARS) coronavirus (Co-V)), were inserted into the vector in frame, and the corresponding recombinant viruses were rescued. The M2 extracellular domain (M2e) is the most promising target for a universal vaccine against type A influenza virus (Fiers et al. 2004 and references therein). Because this 24-amino-acid peptide is not very immunogenic, we fused its gene to that of CTB, a well recognized immune adjuvant (Gonzalea et al. 1993), and inserted the hybrid gene into the PV vector to further boost the immunogenicity of M2e, taking advantage of the ability of live vaccine to stimulate the full immune response in the human body.

The other insert, SARS-RBD, comprises a central fragment (193 amino acid residues) of the S1 subunit of the SARS-CoV S protein and is responsible for viral binding to its receptor on target cells (Wong et al. 2003). The RBD consists of multiple conformational neutralizing epitopes that induce highly potent neutralizing antibodies against SARS-CoV. Rabbits and mice immunized with SARS-RBD produced high titers of neutralizing antibodies against SARS-CoV with a 50% neutralizing titer of 1:10,000 (He et al. 2004; He et al. 2005). Since RBD sequences are relatively highly conserved, recombinant RBD or vectors encoding RBD may be used as safe and efficacious vaccines for preventing infection by SARS-CoVs of various genotypes (Jiang et al. 2005 and references therein).

Materials and methods

PCR amplification of the Sabin 1 poliovirus (PV) genome

Sabin 1 PV is the national reference strain for oral PV vaccine and is stored in our laboratory. We extracted the genomic RNA from Sabin 1 using Trizol reagent (Invitrogen) and reverse-transcribed it using reverse transcriptase (M-MuLV Reverse Transcriptase, RNase H-; New England BioLabs) at 42°C for

60 min with random $(N)_9$ or $poly(T)_{18}$ primers. Three pairs of primers were used to amplify three different genome fragments from the cDNA template, with flanking sequences containing an *Nhe*I site between fragment 1 (f1) and fragment 2 (f2), and a *Bgl*II site between f2 and fragment 3 (f3). The primer pairs used were (shown 5′→3′): f1, AA**GGGCCC**TTAA-AACAGCTCTGGGGGTTGCACCC (forward; *Apa* I restriction site shown in bold) and CAATCATGC-TTTCAAGCATCTGACCTAACC (reverse); f2, GC-GCTTGATGCGAGATACCACACATATAG (forward) and GTTCCTGCTTGATCTTCGAGCGCTT-TGGC (reverse); f3, TCACCTGGTGAAAGCATT-GTGATCGATGGC (forward) and AT**GTC-GAC**CCTCCGAATTAAAGAAAAATTTACCCC-TAC (reverse; *Sal* I restriction site shown in bold). The fragments were separately cloned into pGEM-T-

easy vectors and sequenced. Joining of these fragments according to the strategy shown in Fig. 1 recreated the complete PV cDNA.

Introduction of a polylinker cassette into modified plasmids

The pGEM-f1 plasmid was digested by *Apa*I and religated to remove some restriction enzyme sites, including an *EcoR*I site on the original vector. The religated plasmid was modified further using over-lapping extension PCR to insert a polylinker cassette at the 5′ end (nucleotide (nt) 743) of the polyprotein open reading frame (ORF). Briefly, the PV segment composed of nts 390–743 was amplified using two primers, with the cassette sequence attached to the reverse primer, and the nt 744–890

Fig. 1 Schematic illustration of cloning strategy used to assemble three poliovirus (PV) genome fragments to reconstruct the complete virus genome. Three PV genome fragments (PV-f1, -f2, and -f3) obtained by RT-PCR were cloned separately into pGEM-TEasy vectors, creating three new plasmids. The PV-f2 and PV-f3 fragments were recovered from two of these plasmids and subcloned into the *Nhe*I/*Sal*I site of the third plasmid, pGEM-PV-f1, to create an ∼10-Kb pGEM-TEasy derivative carrying the reconstructed genome
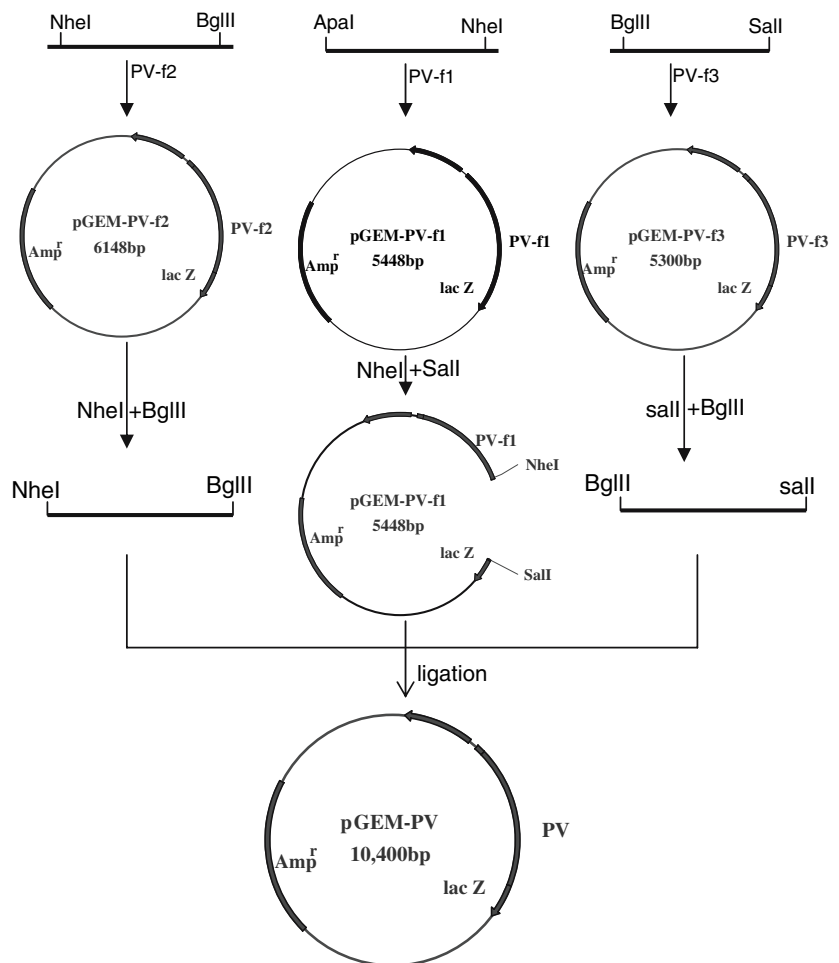
PV fragment was amplified using two primers, with the cassette sequence attached to the forward primer. These fragments were then hybridized to form the nt 390–890 fragment containing the cassette sequence.

This hybrid fragment was digested with *PflM*I (nt 412) and *BstB*I (nt 866) and used to replace the corresponding original fragment in the modified pGEM-f1 plasmid. Similarly, the same cassette was also introduced into pGEM-f2 at the junction between the genes encoding the PV capsid protein VP1 and protease 2A. The cassette contained *EcoR*I, *Xma*I, and *Xho*I sites to facilitate the introduction of extraneous genes and a 3C^{Pro} protease cleavage site to allow the release of the expressed protein. The modified plasmids were named pGEM-f1m and pGEM-f2m, respectively.

Engineering a hammerhead Rz in the vector

The first 300 nts of the PV genome sequence were amplified by PCR using one pair of primers; the sense primer contained an *Apa*I site and an Rz sequence (Herold and Andino 2000). The resulting PCR products were cloned into the pGEM-TEasy vector and digested with *Apa*I and *Pml*I. A 100-bp fragment was recovered and subcloned into *Apa*I/*Pml*I-linearized pGEM-f1 and pGEM-f1m plasmids to yield plasmids pGEM-f1Rz and pGEM-f1mRz, respectively.

Addition of a poly(A) tail

The 400-nt segment at the 3′ end of the PV genome was amplified by PCR using a primer pair, of which the antisense primer contained poly(A)$_{40}$. The PCR products were cloned into pGEM-TEasy and digested with *Sap*I and *Sal*I. A 200-bp fragment was recovered and ligated into *Sap*I/*Sal*I-linearized pGEM-f3 to form plasmid pGEM-f3A.

Vector construction

Using the same genome ligation strategy, the three plasmids and their derivatives were joined to yield vectors carrying various combinations of the Rz, poly(A)$_{40}$, and polylinker motifs. The resulting vectors formed two basic groups; in the PV1 group, the polylinker cassette was in frame with the polyprotein immediately following the initiating AUG at nt 743, whereas in the PV2 group, the polylinker cassette was at the junction between the VP1 and 2A regions starting at nt 3,385 (Fig. 2).

RNA transcription and purification

The recombinant plasmids were linearized with *Sal*I and purified by phenol/chloroform extraction followed by ethanol precipitation. RNA was prepared in a 40-μl volume using a RiboMax Large Scale RNA Production system (Promega) according to the manufacturer's instructions and purified using an SV
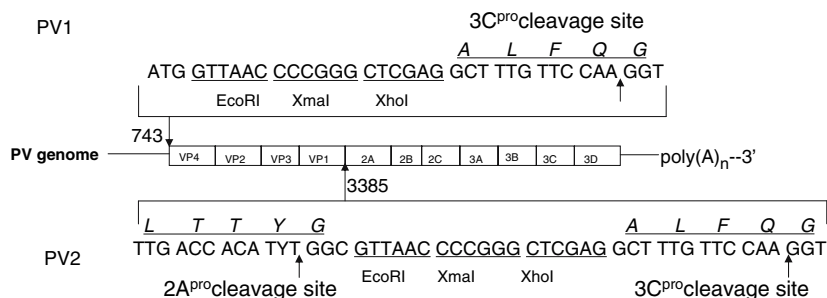

Fig. 2 Schematic diagram of viral genomes of Sabin 1 PV and two vector constructs. Identical polylinker cassettes carrying *EcoR*I, *Xma*I, and *Xho*I restriction sites were inserted either at the 5′ end of the PV open reading frame (PVl) or at the P1–P2 junction (PV2). The vector PVl encodes an engineered cleavage site for 3C protease 3′ to the multiple cloning site (MCS). In the vector PV2, cleavage sites for protease 2A (native) and 3C (engineered) are encoded 5′ and 3′ to the MCS, respectively. The arrow indicates the breakage site by enzyme digestion. The inserts integrated into the MCS were confirmed by RT-PCR with primer sets amplifying nts 500–1,000 (for PV1) and 3,000–3,500 (for PV2). The size of each expected product is 500 bp plus the length of the insert

Total RNA Isolation System (Promega). The purified RNA was eluted in RNase-free water and stored at −70°C.

Rz reaction in vitro

Purified RNA containing the Rz motif was dissolved in reaction buffer (40 mM Tris, pH 7.5, 20 mM $MgCl_2$ in DEPC-treated water) to achieve a final concentration of 0.6 μg μl^{-1}. The mixture was incubated at 37°C for 0.5–2.5 h. Samples were collected at various time points during the incubation and electrophoresed on a 0.8% agarose gel to check the integrity of the RNA and on a 10% polyacrylamide gel to analyze the efficiency of the cleavage reaction.

Transfection of Vero cells

Vero cells were grown to confluence in MEM supplemented with 5% calf serum, collected, and seeded on six-well plates at 2.5×10^5 cells per well. Twenty-four hours later, the medium was removed from the wells, and the cells were washed twice with serum- and antibiotic-free medium. The liposome reagent Tfx-20 (Promega) was used to transfect the cells, according to the manufacturer's instructions. The cells were transfected at 35°C for 1.5 h, and growth medium was added to each well after transfection. The plates remained overnight at 35°C under a 5% CO_2 atmosphere after transfection, and the growth medium was changed on the following day.

The cytopathic effect (CPE) was observed daily to assess rescue of the recombinant viruses. Cells showing CPE were frozen and thawed once to harvest the virus. Titers of viruses in the supernatant fractions of these transfected cultures were subsequently determined using Hep-2 cells. Alternatively, one day after the transfection, the medium was replaced with fresh growth medium containing 1% agarose, and plaque assays were performed as described by Emini et al. (1983) to determine the transfection efficiencies of the various vector RNAs.

Construction and identification of recombinant virus

Two foreign inserts, the 450-bp CTBM2e and 560-bp SARS-RBD gene fragments, were cloned into pGEM-TEasy with *Eco*RI and *Xho*I sites at the 5′ and 3′ ends, respectively. The 380-bp CTB gene (without the stop codon) was amplified from the genome of *Vibrio cholerae* O139, strain 93-3. A 72-bp fragment encoding the M2 extracellular domain (M2e) of type A influenza viruses (5′-ATGAGCCT-TCTAACCGAGGTCGAAACGCCTATCAGAAAC-GAATGGGGGTGCAGATGCAACGATTCAAGTG-AC-3′) was fused to the CTB gene using primer-extension PCR. The SARS-RBD gene fragment was amplified from a plasmid containing the S-protein gene of SARS-CoV using PCR with the forward and reverse primers 5′-ACGTGAATTCAATATTA-CAAACTTGTGTCCTTTTGG-3′ and 5′-ACGTC-TCGAGAACCGTGGCCGGTGCATTTAAAAGT-3′, respectively. Two inserts were transferred into pPV1RzA vectors in frame, and the recombinant virus was rescued according to the established procedures described above.

Serial passage and RT-PCR

Each recombinant virus was consecutively introduced into Vero cells. In each passage, recombinant virus harvested from the previous passage was used to infect cell monolayers at a multiplicity of infection (MOI) of 10. Infected cells were cultured for 24 h, and the supernatant fractions were harvested as a virus source for each passage when full CPE appeared. Total RNA was extracted from virus suspensions, and RT-PCR was performed to amplify the genome segment comprising nts 500–1,000 of the PV genome, which spans the inserted genes, or to amplify the gene with gene-specific primers. As a control, the nt 3,000–3,500 segment of the genome was also amplified.

Expression of foreign proteins by recombinant virus

Expression of foreign proteins was confirmed using an indirect immune fluorescence method. Vero cells plated on cover slips were inoculated with recombinant virus at an MOI of 0.01. After 24 h incubation, the growth medium was removed, and cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature for 10 min. The cells were permeated with 0.5% Triton X-100 in PBS for 15 min and then washed three times with PBS.

The washed cells were incubated with an M2e-specific monoclonal antibody (Ab) prepared in our laboratory and with rat Abs specific for inactivated SARS-CoV (kindly provided by SinoVac Ltd., China). The incubation proceeded for 1 h at 37°C in a PBS buffer supplemented with 3% bovine serum albumin. After three washes with PBS, the cells were stained with FITC-labeled secondary antibodies (Santa Cruz, USA) for 1 h at 25°C in the same buffer. Immunofluorescence staining of the cells was visualized using an Olympus BX51 microscope, and fluorescence images were captured using an adapted digital camera.

Results

Construction of PV vectors

PCR amplification of cDNA using random or poly(T)$_{18}$ primers resulted in synthesis of rcDNA and tcDNA, respectively. The PV-f1 fragment (nts 1–2,510) was obtained using rcDNA as the template, whereas the PV-f3 fragment (nts 5,561–7,441) was obtained using tcDNA as the template. Both cDNA templates yielded the PV-f2 fragment (nts 2,431–5,640), but the tcDNA template generated more PCR products than did rcDNA. These three fragments were recovered from an agarose gel and cloned into the pGEM-T vector, yielding pGEM-f1, -f2, and-f3, respectively. Comparing these sequences with the Sabin 1 genome sequence (GenBank accession no. AY184219), we identified two silent mutations

(26A→G and 355C→T) and one non-silent mutation (6735A→G); the latter mutation resulted in a Lys^{250}→Arg substitution in RNA-dependent RNA polymerase (polymerase 3D). The structures of the pGEM-f1, -f2, and -f3 plasmids and their derivatives were confirmed by enzyme digestion and sequencing. Various combinations of elements from these vectors were used to create a total of 8 new vectors following the assembly strategy shown in Fig. 1 (Table 1).

Rz reaction in vitro

Samples were collected for analysis at various time points. The cleaved 52-bp Rz sequence was analyzed by PAGE using 15 µg of total RNA per well to make the Rz fragment visible. The cleavage reaction was time-dependent and reached a plateau at 1.5 h (Fig. 3). Electrophoresis of the RNA on a 1% agarose gel showed that the integrity of the RNA was not compromised at the time of observation. Therefore, the RNA Rz reactions were subsequently performed for 1.5 h at 37°C.

Transfection of Vero cells

Vero cells were transfected with various amounts of RNA. To minimize the toxicity effect to the cells, 5 µl of Tfx-20 were used. The transfection results are summarized in Table 1, which shows that only RNAs from vectors containing both the Rz and poly(A) tail motifs, and subjected to 37°C treatment, had the highest transfection efficiency. For example, 1 µg of RNA derived from pPV1RzA or pPV2RzA yielded

Table 1 Infectivity of RNAs derived from various vectors

Vector	Rz	Poly(A)$_{40}$	PFU (µg RNA)$^{-1}$	Time to observe CPE(d)	
				10 µg RNA	1 µg RNA
pPV1	−	−	0	/	/
pPV1Rz	−	+	0	/	/
pPV1A	+	−	1.1–2.1[a]	6	/
pPV1RzA	+	+	500–1,200	2.5	4
pPV2	−	−	0	/	/
pPV2Rz	−	+	0	/	/
pPV2A	+	−	0.3–1.2[a]	6	/
pPV2RzA	+	+	200–800	2.5	4

+, presence; −, absence ; /, no CPE
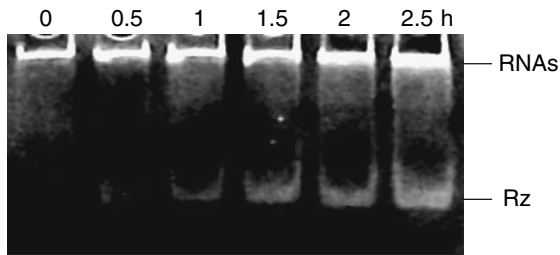
[a] Result obtained using 10 µg RNA

Fig. 3 Time course of in vitro Rz reaction of synthesized RNA. The reaction mixtures were analyzed by 10% PAGE to assess the amount of Rz cleavage from the synthesized RNA at various time points. At 52 bp, Rz migrates very close to bromophenol blue, whereas the larger RNA molecules remain close to the sample wells

500–1,200 and 200–800 plaque-forming units (PFUs), respectively.

The more the amount of RNA used for transfection, the earlier CPE appeared. For example, CPE was very significant at 60 h after transfection with 10 µg of pPV1RzA RNA, but when only 1 µg of RNA was transfected, CPE was not observed until more than 5 days had passed. Vectors carrying only poly(A)$_{40}$ also produced infectious clones, albeit with lower efficiency. For example, 10 µg of pPV1Rz RNA were needed to produce dozens of infectious clones, and CPE appeared at 6 d. For the remaining vectors, CPE of the transfected Vero cells did not occur within 8 days post-transfection, even with as much as 30 µg RNA (not shown).

Rescue of recombinant virus using the established vector

Two foreign antigen genes, CTBM2e and SARS-RBD, were cloned into the pPV1RzA vector using

established procedures to produce the recombinant viruses rPV-CTBM2e and rPV-SARS-RBD, respectively. RT-PCR and sequencing results confirmed that the rescued viruses contained the correct inserts.

Genetic stability of recombinant viruses

The genetic stability of recombinant viruses rPV-CTBM2e and rPV-SARS-RBD was examined by RT-PCR (and sequencing, if necessary) over 12 consecutive passages of each virus. Virus rPV-CTBM2e retained the intact insert without any truncations over the entire period of 12 passages. On the other hand, virus rPV-SARS-RBD was not stable during passage, as shown by a gradual accumulation of a shorter band that derived from the vector (as shown by sequencing). At the sixth passage, only a small fraction of the rPV-SARS-RBD virus mixture contained the SARS-RBD gene, and no insert remained after eight passage cycles (Fig. 4).

Expression of foreign antigens in Vero cells

Recombinant virus-directed expression of foreign antigens in Vero cells was confirmed by indirect fluorescence immunoassays. The SARS-RBD and CTBM2e proteins were detected by using anti-SARS-CoV polyclonal Abs and an anti-M2e monoclonal Ab, respectively, with FITC-labeled secondary Abs (see Materials and Methods). Significant fluorescence signals appeared in both cases, primarily in the cell cytoplasm (Fig. 5). As a plus-strand RNA virus carrying one ORF, the genome of PV was used as the mRNA to direct the synthesis of a large polyprotein. Proteases 2A or 3C are active, even within the
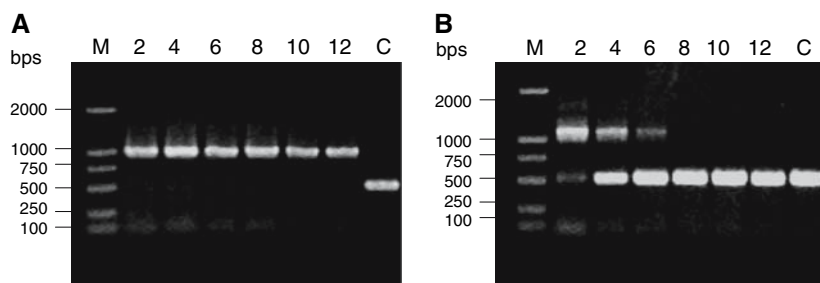

Fig. 4 Genetic stability of recombinant PVs. The recombinant viruses were tested for genetic stability in Vero cells by RT-PCR amplifying Sabin 1 nts 500–1,000, which flanks the foreign inserts. (**A**) RT-PCR patterns of rPV-CTBM2e during

consecutive passages. (**B**) RT-PCR patterns of rPV-SARS-RBD during consecutive passages. Lanes: M, DL2000 markers; C, nts 3,000–3,500 of the recombinant virus (amplified as control)
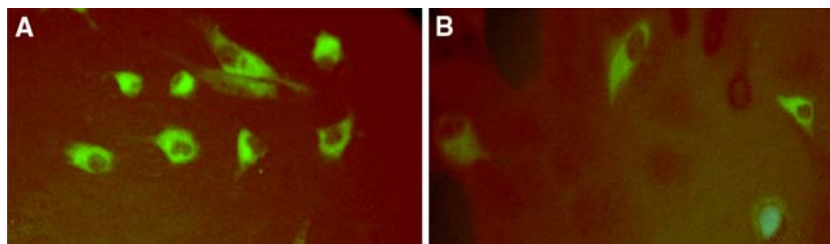
Fig. 5 Expression of foreign proteins by recombinant virus in infected Vero cells. Vero cells grown on cover slips were infected with the recombinant viruses at an MOI of 0.01 and incubated for 24 h. Cells infected with rPV-CTBM2e and rPV-SARS-RBD were stained using anti-M2e monoclonal Ab (hybrid cell supernatant at 1:10 dilution) and mouse anti-SARS-RBD polyclonal Ab (1:50 dilution), respectively, followed by detection with FITC-labeled goat anti-mouse Ab. (**A**) rPV-SARS-RBD-infected Vero cells; (**B**) rPV1-CTBM2e-infected Vero cells. The fluorescence signal is visible primarily in the cytoplasm in both cases

polyprotein, and cleave the polyprotein into its component functional and structural proteins (Racaniello 2001); thus, the foreign antigen with the introduced cleavage site was also released from the polyprotein.

Discussion

In this work, we investigated the possibility that attenuated strains of poliovirus (PV) might be used to construct a vector with high transfection efficiency. Although the value of the oral live PV vaccines (Sabin vaccines types 1, 2, and 3) is widely recognized, these vaccines do have an inherent risk; even though the likelihood is very low, the attenuated live virus can revert to the neurovirulent phenotype. Indeed, a very small number of cases of vaccine-associated paralytic poliomyelitis (VAPP) have occurred in recipients of oral PV vaccine (Melnick 1984). Experimental evidence strongly suggests that most of these cases are caused by Sabin vaccines types 2 and 3 (Kew et al 1981; Minor 1982). In an enquiry made by the World Health Organization, VAPP cases due to type 3 virus were found to be the most common in both vaccine recipients and their contacts. Type 2-induced VAPP occurred more often in contacts than in vaccinees, and Sabin 1 was rarely implicated (Esteves 1988). Given that a human vaccine must be safe for use in human immunization, we selected the Sabin 1 virus as posing the least risk to its recipients.

We cloned the whole genome of Sabin 1 PV by RT-PCR. Sequence comparisons of the resulting clone and the Sabin 1 genome sequence (GenBank accession no. AY184219) revealed only three, single-base mutations. Two mutations were silent, whereas the third one resulted in a Lys^{250} (our sequence)→Arg change in polymerase 3D. A mutation at this third site was also found in another Sabin 1 genome sequence (Genbank accession no. V01150), with a Lys^{250} (our sequence)→Glu change in polymerase 3D. These observations suggest that this site is a mutation "hot spot" in Sabin 1 strains, and the resulting mutations have no significant effect on the function of polymerase 3D.

We also found that pGEM-PV replicated weakly in bacteria. Therefore, large-scale bacterial culture was required for high plasmid yield. When carrying the whole PV genome, the pGEM plasmid appears to become a stringent plasmid that is relatively stable in bacteria. As a result, transfer of the PV genome DNA to a low-copy-number plasmid, like pBR322, was unnecessary. In a previous report, however, Racaniello (Racaniello and Baltimore 1981) expressed a preference for a more stringent vector for cloning PV cDNA.

The HeLa and Hep-2 cell lines are sensitive for PV culture. However, because we were aiming to establish a human vaccine, we selected Vero cells to rescue the recombinant virus. Vero cells are used widely as the cell substrate for preparing human vaccines, including inactivated polio vaccine and rabies vaccine (Montagnon et al. 1981, 1999). Our initial attempts to transfect the Vero cells with RNA derived from PV constructs carrying the polylinker cassette failed to produce infectious clones, despite our use of several different methods, including liposome transfection, electroporation, and calcium

phosphate precipitation. This result suggested that the original vector did not match the Vero cell and needed further optimization.

The two G nts immediately following the T7 promoter are important for efficient in vitro transcription of RNA by T7 RNA polymerase. However, insertion of these two G's places extra nucleotides at the 5′ end of the PV genome, which is a problem, because the authentic 5′ end is essential for viral replication and plus-strand synthesis (Herold and Andino 2000). Thus, we further modified the vector by inserting a hammerhead Rz sequence between the T7 promoter and the virus genome. This construct was designed so that the precisely correct PV 5′ end would be produced after the self-cleavage of the Rz.

Because the Rz reaction can occur during the process of transcription, it releases the Rz moiety to some extent. For maximum cleavage of the Rz motif, we purified the RNA and placed it in a buffer containing 20 mM Mg^{2+}, which is important for Rz activity. To determine the optimal incubation period for the reaction, we examined the reaction progress and the integrity of large RNA molecules at various time points. As discussed below, maximum cleavage of Rz in vitro is necessary for efficient transfection.

A poly(A) tail is believed to stabilize mRNA in mammalian cells. PV carries a poly(A) tract of 75–200 residues at the 3′ end of its genome (Spector and Baltimore 1975). When the poly(A) tract is removed from the PV RNA, the virus is no longer infectious (Spector and Baltimore 1974). In the present study, we also found that a poly(A) tract was necessary for the infectivity of our vectors in Vero cells. However, the Rz element can significantly improve transfection efficiency in Vero cells, with less RNA and shorter incubation periods needed to produce CPE. For instance, 10 μg of pPV1A RNA were needed to produce CPE after 5 d, whereas 10 μg of pPV1RzA RNA achieved significant CPE within 60 h.

Liposome reagents are commonly used for transfection of DNA or plasmids into mammalian cells. For optimized transfection, the charge ratio of reagents to DNA or RNA is an important parameter; this ratio is usually kept as 2:1 to 4:1 to ensure that the complex carries a net positive charge. This facilitates the interaction of the vesicles with the negatively charged target-cell surface. In this study, however, more RNA was needed for optimal transfection efficiency. The RNA: Tfx-20 ratio used was 3:1.

Two foreign inserts, CTBM2e (450 bp) and SARS-RBD (560 bp), were cloned into the pPV1RzA vector in frame, and the recombinant viruses were rescued using the same procedure as that used for the vectors. The successful rescue of both recombinant viruses in this study indicated that the established vectors still worked well when carrying the foreign genes. Both recombinant viruses expressed significant amounts of foreign antigen in the Vero cell cytoplasm, as confirmed by immunofluorescence detection. This observation is consistent with the PV life cycle, as PV completes its replication cycles in the cytoplasm. The gene fragment was expressed with the rest of the PV genome as part of the polyprotein and was cleaved away from the polyprotein by PV-encoded proteases, which cleaved at engineered proteolysis sites flanking the gene insert. The foreign antigens, as well as the viral proteins, released into the cytoplasm could be processed further and presented by the infected cells to stimulate an immune response against the recombinant virus, which is the basis of the polyprotein strategy for modifying PV to be a vaccine vector (Mattion et al. 1994). However, the immune responses against the foreign proteins need further investigation in transgenic mice carrying the human PV receptor (Tang et al. 1997) or in cynomolgus macaques (Crotty et al. 1999).

One of the most important obstacles to success with the polyprotein fusion strategy has been the genetic instability of the recombinant PVs. Previous reports have suggested that this genetic instability is associated with a limitation on insert size and/or genetic recombination within (Tang et al. 1997) or between sequences during minus-strand synthesis (Muller and Wimmer 1998). Nevertheless, a clear molecular mechanism for the control of insert stability has not been well established.

In the present study, rPV-CTBM2e carrying a 450-bp insert was very stable over 12 passages, whereas the 600-bp insert in rPV-SARS-RBD was lost within the first few passages. Even plaque-purified rPV-SARS-RBD with the intact insert remained unstable over three further passages, suggesting that this foreign gene has inherently destabilizing attributes. Lee et al. (2002) reported that the genetic stability of recombinant PV is strongly associated with the G/C content and G/C distribution pattern in foreign inserts of less than 450 bp, and that the genetic instability of foreign inserts can be promoted by increasing the G/C

content and/or replacing the local A/T-rich region with a G/C-rich codon. In our study, however, rPV-CTBM2e remained stable, although the G/C content of CTBM2e is low (36.9%). Both the large size (600 bp) and low G/C content (38%) of the SARS-RBD may contribute to the instability of the rPV-SARS-RBD virus. Further work is needed to increase the stability of this recombinant virus.

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